

## **Myoblast Models of Skeletal Muscle Hypertrophy and Atrophy**

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## **Abstract**

Purpose of Review: To highlight recent breakthroughs and controversies in the use of myoblast models to uncover cellular and molecular mechanisms regulating skeletal muscle hypertrophy and atrophy.

Main Findings: Myoblast cultures provide key mechanistic models of the signalling and molecular pathways potentially employed by skeletal muscle *in-vivo* to regulate hypertrophy and atrophy. Recently the controversy as to whether IGF-I is important in hypertrophy following mechanical stimuli vs. alternative pathways has been hotly debated and is discussed. The role of myostatin in myoblast models of atrophy, and interactions between protein synthetic pathways including Akt/mTOR and the ‘atrogenes’ are explored.

Summary: Targeted *in-vivo* experimentation directed by skeletal muscle cell culture and bioengineering (3-dimensional skeletal muscle cell culture models) will provide key biomimetic and mechanistic data regarding hypertrophy and atrophy and thus enable the development of important strategies for tackling muscle wasting associated with ageing and disease processes.

**Keywords:** muscle precursor cell, satellite cell, IGF-I, myostatin, 3D muscle constructs.

## **Introduction: The use of Myoblast Models to Investigate Mechanisms of Skeletal Muscle Size Regulation**

Adult skeletal muscle fibre number is set *in-utero* and adult fibres are terminally differentiated or incapable of division. Despite these phenomena, adult skeletal muscle is highly adaptable, responding to the soluble and biophysical cues that it encounters on a daily basis. Skeletal muscle undergoes rapid growth (hypertrophy) during development, exercise, stretch and mechanical loading (weight bearing) and also severe loss (atrophy) with ageing, disuse and disease. It is also capable of repair and regeneration following injury. Much of this adaptability (growth and repair) is achieved via resident adult stem cells, termed satellite or muscle precursor cells (MPC) that have mitotic potential. Recent data suggest that blocking myostatin (a negative regulator of muscle mass), enables hypertrophy not only via satellite cell accretion but also via increased modulation of the synthesis and turnover of structural proteins within a myotube/fibre (1)\*.

Satellite cells are fundamental to muscle adaptation; they are specialised cells, normally residing in a quiescent state beneath the basal lamina of myofibres. Following physiological, bio-mechanical or pathological cues, they are activated to myoblasts and become committed to the muscle/mesenchymal lineage, adopting not only a muscle phenotype, but given relevant cues, also neural (2), osteoblast (3, 4), chondrocyte (5) and adipocyte (6) lineages. In order to elicit reparative responses, satellite cells must **i.** be activated, **ii.** increase their numbers (proliferation), **iii.** migrate to the site of injury and **iv.** fuse (differentiate) with the damaged fibre (7). Because of their fundamental roles in muscle maintenance and adaptation, myoblasts are frequently studied as *in-vitro* models of growth, migration, differentiation and

death. Basal, hypertrophic or atrophic conditions are used and provide relevant models of muscle growth (exercise and puberty) or wasting in catabolic conditions (e.g. cancer, AIDS, congestive heart failure, sepsis, COPD, rheumatoid arthritis and sarcopenia (muscle wasting associated with ageing)). These myoblast models use either primary skeletal muscle cells derived from human or animal biopsies, or, more frequently, established cell lines such as the mouse C<sub>2</sub>, C<sub>2</sub>C<sub>12</sub> (a clonal derivative and daughter of the C<sub>2</sub> cells), or rat L6 cells. Advantages of cell lines vs. primary human culture include: Availability, ethics, reduced cost, access to cells, speed of growth and pure myogenic populations. Disadvantages of muscle cultures vs. *in-vivo* models include: Reduced differentiation capacity with passage, an inability to sustain myotubes for extended periods in culture and the environment of 2-D monolayer that lacks bio-mimicry, thus making direct comparisons with muscle *in-vivo* difficult. Development and manipulation of models using both collagen and fibrin gels to incorporate a 3-Dimensional (3-D) structure for myoblasts in culture (8)\*\*(9) will potentially enable more physiological experimentation and analyses of the regulators of hypertrophy and atrophy.

This review will therefore highlight the most recent breakthroughs in the use of myoblasts as models to investigate and manipulate cellular and molecular regulators of hypertrophy and atrophy. Given the nature of this review, *in-vivo* data will be reported only to portray the relevance of the *in-vitro* findings.

## **Myoblast Models of Hypertrophy: Insulin-like Growth-Factors (IGFs) versus Mechano-Transduction.**

Insulin-like Growth Factors (IGFs) influence hypertrophy of skeletal muscle primary and cell line cultures, enhancing proliferation, differentiation (reviewed in (10)), survival (11), satellite cell recruitment (12) and myofibrillar protein accretion (13). Despite high levels of systemic liver-derived IGF, a recent *in-vivo* study suggests that local production of IGF-I by skeletal muscle is imperative in hypertrophy. Where liver deficient IGF-I mice demonstrate similar strength gains to controls with larger increases in IGF-I receptor tyrosine phosphorylation (14) and associated PI3K/Akt/mTOR induced protein synthesis (14). Despite these compelling data, the importance of IGF-I in mechanical load (weight bearing) induced hypertrophy has recently been debated (15, 16)\*\*. Never the less, the withdrawal of serum *in-vitro* is sufficient to induce myoblast differentiation in both cell lines and primary cultures which are able to produce endogenous IGF-I that facilitates the fusion process (17)\*\*; furthermore, supplementation with IGF-I augments hypertrophy compared with basal conditions (18-20). Validation of the mechanisms pertaining to IGF, hypertrophy and mechanical load *in-vivo* therefore continues and would be facilitated by an *in-vitro* model that incorporates mechanical load. A recent study, using stretch, illustrated that primary chick myotubes undergo significant hypertrophy following activation of the PI3K/Akt/mTOR pathways (which lie downstream of IGF-I/IGF-IR activation). Hypertrophy was also prevented using pharmacological inhibitors of PI3K and mTOR (21), however, the authors did not measure endogenous production of IGF-I. Therefore, explicit confirmation of the role of IGF in these models is awaited.

## **Mechano-Transduction Signalling**

The mammalian target of rapamycin, mTOR, can be activated via signals independent of IGF-I, via a pathway involving phospholipase D (PLD), phosphatidic acid and a downstream regulator Rheb (ras homologue enriched brain) (see (22-24). Furthermore, mTOR can be stimulated via amino acids, particularly leucine (25, 26), potentially via Rheb binding and interaction with amino acid sensitive phosphatidylinositol 3-kinase/Vps34 (24) or MAP4K3 (27). The stress response gene Redd 2, may also be important in inhibiting mTOR via the tuberous sclerosis 1 (TSC1) and 2 (TSC2) complex (28), and following mechanical overload Redd 2 is reduced to enable mTOR to initiate p70S6K expression, which is involved in protein synthesis and hypertrophy (29) (see Figure 1). To substantiate that mechanical signals can operate independently of IGF-I signalling; *in-vivo* evidence from Spangenburg *et al.* (30)\*\* suggests that mice overexpressing a dominant negative form of IGF-IR (MKR mice), elicited similar hypertrophic responses, following synergistic ablation of the plantaris muscle compared to wild-type mice. Suggesting that IGF-I is not required for load-induced hypertrophy. By contrast, Heron-Milhavet *et al.* (31)\*\* also using the MKR mouse showed IGF-I to be fundamental in myoblast fusion, with primary MKR myoblasts showing impaired differentiation, versus wild-type controls, following damage (31). Interestingly, MKR-derived muscle cells had equivalent levels of myogenin (a myogenic regulatory factor fundamental to lineage and hypertrophy) positive cells to wild type. However, the ability of the myogenin positive cells to fuse into multinucleated myotubes was significantly lower in MKR vs. wild-type-derived myoblasts. Indeed, a significantly greater proportion of fusion-hampered MKR-derived myoblasts compared with control cells (31), suggests that IGF-I does play an influential role in differentiation and hypertrophy but that other

factors enable hyperplasia. Although data from Spangenburg *et al.* (30) appear to contradict the observations by Heron-Milhavet *et al.* (31), different modes of hypertrophy were being examined. Spangenburg *et al.* (30) performed no cellular or histological analyses, thus questioning whether the increase in muscle mass observed in MKR mice (similar to controls) corresponded to a true hypertrophic vs. hyperplastic responses.

### **A Biphasic Role for IGF-I?**

Utilising an *in-vitro* model of hypertrophy and atrophy (comparing younger phenotypes of clonally derived daughter C<sub>2</sub>C<sub>12</sub> vs. parental C<sub>2</sub> cells), we have recently published that IGF-I is important for the greater differentiation potential of C<sub>2</sub>C<sub>12</sub> vs. C<sub>2</sub> cells (17)\*\*. Importantly, IGF-I expression was similar at 48 hrs following initiation of differentiation in both cell types, despite greater morphological differentiation in the C<sub>2</sub>C<sub>12</sub> cells. By 72 hrs, however, IGF-I expression was significantly greater in C<sub>2</sub>C<sub>12</sub> vs. C<sub>2</sub> cells as were morphological and biochemical differentiation. These data indicate a potential biphasic role for IGF in underpinning the temporal complexity of differentiating myoblasts. Despite similar levels of IGF-I expression at 48 hrs, reductions in myoD and myogenin were evident in the C<sub>2</sub> vs. C<sub>2</sub>C<sub>12</sub> cells and this may underpin the reduced potential for differentiation of these cells. Finally, an inverse expression pattern of IGF binding protein-2 (IGFBP2) was evident in the two cell types. The role for IGFBP2 warrants further investigation as it may be crucial in modulating IGF-induced differentiation especially with age (17)\*\*.

### **Other Potential Hypertrophic Mechanisms**

A recent study, using a single fibre approach, suggested that extracellular matrix and fibroblasts are fundamental for muscle hypertrophy, enabling increases in hepatocyte growth factor (HGF) expression. HGF binds to the c-met receptor on the cell membrane of skeletal muscle, thus enabling satellite cell activation (32). Importantly, however, high levels of HGF are not only associated with satellite cell activation, but also the up-regulation of myostatin (discussed below) mRNA, the product of which leads to satellite cell quiescence. These data suggest a fine regulatory role for HGF, distinct from IGF/mTOR signalling, in hypertrophy vs. self-renewal of skeletal muscle cells (33)\*. Nitric oxide (NO) is also reportedly increased following mechanical stretch and leads to the up-regulation of matrix metalloproteinase activity, enabling matrix remodelling required to support hypertrophy (32). Indeed, overexpression of MMP-9 in C<sub>2</sub>C<sub>12</sub> cell clones (C2M9) improves their migration *in-vitro* and their engraftment *in-vivo*, both of which are required for hypertrophy and regeneration (34)\*.  $\beta$ -catenin/c-Myc-signaling, important in ribosomal biogenesis, also increases following mechanical overload (load on the muscles, which leads to failure), with inactivation of  $\beta$ -catenin completely preventing hypertrophy in response to mechanical overload in mice (35). Indeed, hypertrophy induced in C<sub>2</sub>C<sub>12</sub> myoblasts using both IGF-IEa and MGF increased nuclear  $\beta$ -catenin *in-vitro* (36) implicating a role for this molecule in potentially linking hypertrophy following IGF signalling and/or following a mechanical stimulus.



## **MicroRNAs at the Cutting Edge**

The class of approximately 22 nucleotide noncoding RNAs (microRNAs) that regulate gene expression at the post-transcriptional level may play fundamental roles in skeletal muscle hypertrophy. Recently, both miR-1 and miR-206 have been implicated in skeletal muscle cell differentiation. Overexpression in C<sub>2</sub>C<sub>12</sub> myoblasts reduced proliferation and induced differentiation *in-vitro* (37)\*. These miRNAs also function to control among other regulators, Pax7, which is required for appropriate satellite cell survival, proliferation, and differentiation. The role of miRNAs in a myoblast model of mechanical load requires further investigation.

## **Summary: Myoblast Models of Hypertrophy**

Overall, the convergence of mechanical, endocrine, autocrine and paracrine signals results in activation of PI3K, Akt, mTOR leading to protein synthesis and hypertrophy via proliferation and differentiation of myoblasts, as well as corresponding ribosomal biogenesis through  $\beta$ -catenin/c-Myc-signaling. However, the relative contribution of each parameter, especially following mechanical load remains to be determined and has implications for therapeutic interventions aimed at improving hypertrophy during disease, ageing and following exercise. Finally, the importance of the implementation of 3-D myoblast models to study the integration of skeletal myoblasts with the ECM *in-vitro*, and to apply to situations of mechanical load/overload or stretch *in-vivo* are important for future developments in the field.

## **Muscle Atrophy: The Problem**

Skeletal muscle atrophy occurs when proteolysis overwhelms protein synthesis. Increased protein degradation may occur as a consequence of many factors, including changes in anabolic hormones e.g. IGF-I, GH, testosterone, glucocorticoids; and increases in TGF- $\beta$ , myostatin, and cytokines such as TNF- $\alpha$ , TWEAK and IL-6. Oxidative stress and reduced amino acid availability can also tip the balance in favour of atrophy. Muscle wasting can occur as a consequence of: mechanical unloading, a reduction in use/exercise (disuse atrophy), chronic catabolic disease (cachexia) and ageing (sarcopenia). Even though resistance exercise may slow the atrophy process, many patients are too old, ill or simply unable (frail or obese) to exercise. Furthermore, resistance exercise has to be continually undertaken to be of long-term benefit, meaning high cost of skilled trainers and high drop-out due to its demanding physical nature. It is therefore important to also develop pharmacological therapies to treat muscle atrophy.

## **Myostatin and Atrophy**

It is beyond the scope of this review to discuss all factors that contribute to muscle atrophy (for excellent current reviews see (7, 38, 39)). However, recent *in-vitro* myoblast research has focussed on myostatin (growth differentiation factor- 8/GDF-8). Myostatin is a member of the Transforming Growth Factor-Beta (TGF- $\beta$ ) family of proteins and a negative regulator of skeletal muscle growth. Pioneering work by McPherron and colleagues (40, 41) using knock out technologies, demonstrated the important inhibitory role of myostatin in skeletal muscle of mice and also reported that the ‘double muscling’ phenotype in Belgian Blue and Piedmontese cattle occurred as a result of mutations in the myostatin gene (41). As a consequence, this

inhibitory growth factor has received a lot of attention as a potential therapeutic target to combat muscle wasting. Myostatin<sup>-/-</sup> mice that are crossed with follistatin transgenic mice display even larger muscle phenotypes as a result of blocking other inhibitory TGF- $\beta$  family members such as GDF-11 and activins (42). However, the first human trial using low dose anti-myostatin antibodies in muscular dystrophy patients did not enable hypertrophy (43). However, this is a complex disease, where the underlying disorder is due to a lack of dystrophin rather than an increase in myostatin and it may not be the best model (44).

### **Myostatin Signalling**

As a consequence of these data, mouse and rat myoblasts have been utilised to investigate the molecular mechanisms of myostatin in muscle. Myostatin reportedly blocks differentiation of myoblasts into myotubes (45) by reducing myoD (46), myogenin and protein synthetic pathways via Akt in C<sub>2</sub>C<sub>12</sub> myotubes (47) and via Akt/TORC1/p70S6K in human skeletal myoblasts (48)\*\*. Myostatin signals via the type IIb activin receptor that enables interactions with activin receptor-like-kinase 4 (ALK4) or ALK5 (both type I receptors- see Figure 1) (49). As a consequence of the association of these myostatin/receptor complexes, phosphorylation of transcription factors Smad2 and Smad3 occurs followed by their translocation to the nucleus (50) where they alter gene transcription. Trendelenburg *et al.* (48)\*\*, demonstrated that follistatin (myostatin inhibitor) and type I ALK receptor inhibitors increased both the size and number of human skeletal myoblasts in culture and, in the presence of exogenous myostatin were able to restore differentiation capacity. Furthermore, siRNAs for Smad 2 or 3 reduced the effect of myostatin on differentiation, with both in combination eliciting an additive effect. Interestingly, there was a 50% reduction in

phosphorylated Akt and p70S6K in the presence of myostatin in differentiating myoblasts and exogenous IGF-I could rescue this effect. However, IGF-I did not change Smad2/3 reporter activity indicating that IGF-I did not oppose myostatin actions via Smad, but via Akt and the induction of protein synthesis via p70S6K. Overall, therefore the IGF-I/Akt/protein synthesis signalling seems dominant over the myostatin/Smad inhibition. Conversely however, Smad 2 or 3 siRNAs restored Akt activation in the presence of myostatin, suggesting Smad2/3 do regulate Akt function but distinctly to IGF-I (48)\*\*.

A recent study by Satori *et al.* (51)\*\* published simultaneously with that of Trendelenburg *et al.* (48)\*\* showed that activation of the Smad 2 and 3 pathway using electroporation to introduce genes encoding active forms of ALK4 or 5 and TGF- $\beta$  itself, induced myofibre atrophy. This effect could be reversed using small hairpin RNAs (shRNAs) blocking Smad2 and Smad3. Importantly, constitutive overexpression of Akt prevented the muscle fibre atrophy induced by Smad2/3 activation (electroporation for ALK4 or 5 mentioned above), further co-borating the *in-vitro* role of Akt in reducing the impact of myostatin.

### **Myostatin: Protein Synthesis or Protein Degradation?**

Some controversy remains over whether myostatin functions via traditional expression of “atrogenes” that promote protein degradation via E3 ubiquitin ligases such as MuRF1 and MAFbx. Early work strongly suggested that myostatin increased levels of FOXO1 that in turn up-regulated MAFbx (47) that leads to protein degradation of cytoskeletal proteins such as desmin and titin. Similarly in C<sub>2</sub>C<sub>12</sub> myoblasts the addition of myostatin increased MAFbx, but not MuRF1. Data were

confirmed in murine models where myostatin increased MAFbx but not MuRF1 expression (51)\*\*. By contrast, the study by Trendelenburg *et al.* (48)\*\* reported a decrease in both MuRF1 and MAFbx mRNA. However, Welle (52) reviewed that neither publication included a direct measure of proteolysis, however, that the vast majority of evidence suggests that changes in protein synthesis rather than degradation are key. Although compelling *in-vitro/in-vivo* signalling evidence suggests reduced protein synthesis, unresolved studies regarding the protein degradation remain. Indeed, very recent findings suggesting that the *in-vivo* murine or *in-vitro* myoblast knockdown of MAFbx, using shRNAs, suppresses myostatin expression and muscle atrophy (53)\*\*, suggesting a feed-forward loop whereby increased MAFbx influences the local production and hence action of myostatin.

### **Myostatin and Premature Ageing?**

Although myostatin inhibitor studies have shown some success in reducing wasting in rodents (54, 55), myostatin inhibitors in human studies should be approached with care when considering regeneration with age. McFarlane *et al.* (56) showed that blocking myostatin, causes high Pax7 expression resulting in increased self-renewal of C<sub>2</sub>C<sub>12</sub> myoblasts followed by quiescence. However, they also reported that over expressing Pax7 in C<sub>2</sub>C<sub>12</sub> cells conferred increased self-renewal but reduced myogenic proliferation and differentiation. Therefore, blocking myostatin in adults may be advantageous in the short-term; however, high expression of Pax7 would influence self-renewal and differentiation and potentially affect subsequent regeneration in later life. This may further compound ageing where myostatin levels are already higher than in younger individuals (57). Indeed, myostatin knock out animals, although displaying larger muscle mass, are not proportionally stronger (58),

this too would be detrimental, i.e. increased weight, but not strength to lift in older people.

## **Conclusion**

Myoblast models have paved the way for understanding the convergence of key mechanisms involved in hypertrophy and atrophy of skeletal muscle; some of the most pertinent recent findings have been discussed in this review. However, future development of myoblast models must incorporate engineering strategies to make the models more reflective of the *in-vivo* situation and evolve the current 3-D models already available (8)\*\*(9). In this way, cell-based models in a dish can be utilised to address key *in-vitro* questions, which can then be focussed to address more challenging *in-vivo* questions.

## References

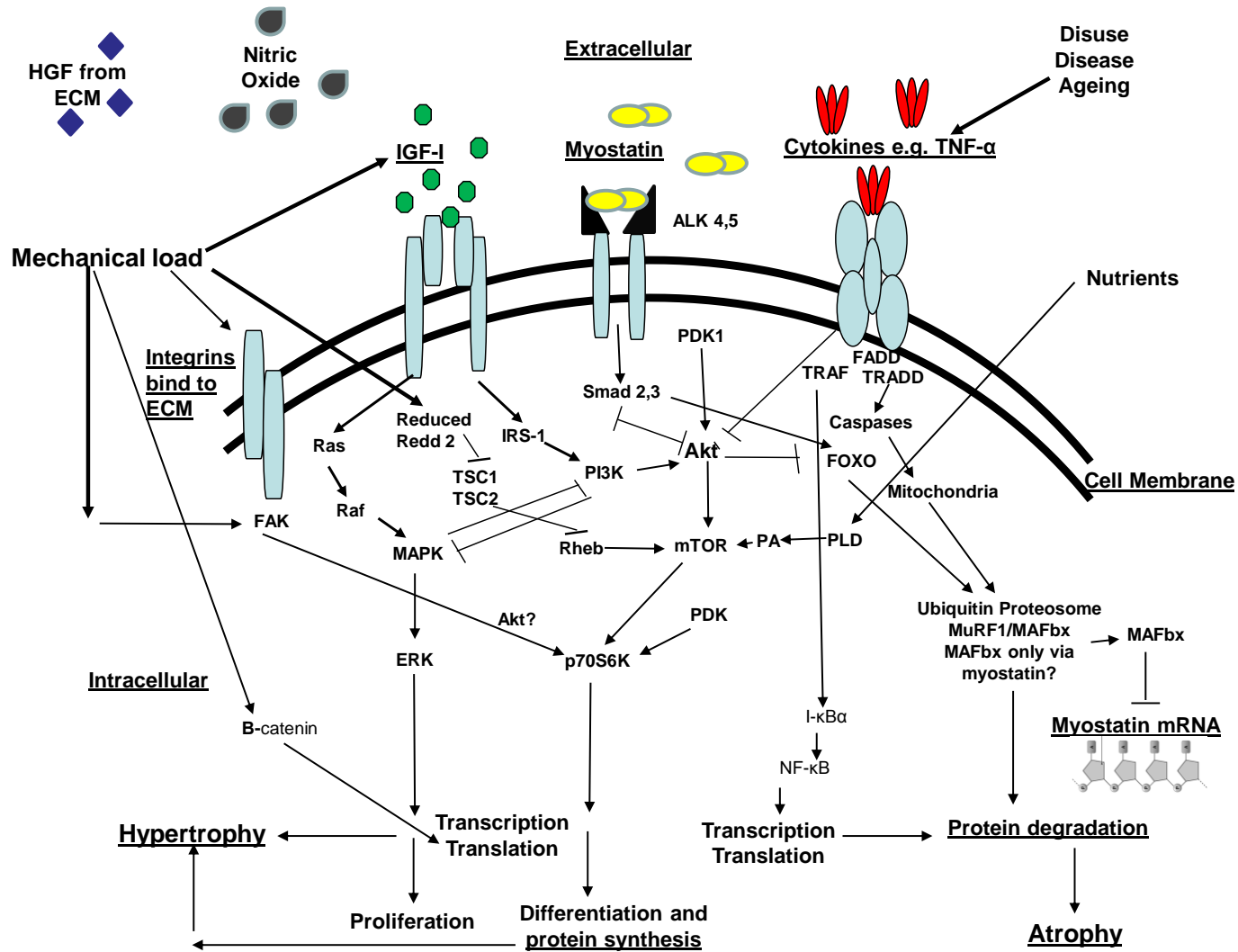
1. Amthor H, Otto A, Vulin A, Rochat A, Dumonceaux J, Garcia L, et al. Muscle hypertrophy driven by myostatin blockade does not require stem/precursor-cell activity. *Proc Natl Acad Sci U S A*. 2009 May 5;106(18):7479-84.
2. Schultz SS, Lucas PA. Human stem cells isolated from adult skeletal muscle differentiate into neural phenotypes. *J Neurosci Methods*. 2006 Apr 15;152(1-2):144-55.
3. Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, et al. Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol Cell Biol*. 2000 Dec;20(23):8783-92.
4. Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, et al. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *J Cell Biol*. 1994 Dec;127(6 Pt 1):1755-66.
5. Peng H, Huard J. Muscle-derived stem cells for musculoskeletal tissue regeneration and repair. *Transpl Immunol*. 2004 Apr;12(3-4):311-9.
6. Lee SJ, Lee EJ, Kim SH, Choi I, Lee DM, Lee HJ, et al. IL-17A promotes transdifferentiation of mouse myoblast cells (C2C12) into adipocytes by increasing the expression of peroxisome proliferator-activated receptor gamma through CAAT/enhancer binding protein beta signaling. *Biotechnol Lett*. 2010 Oct 20.
7. Saini A, Faulkner S, Al-Shanti N, Stewart C. Powerful signals for weak muscles. *Ageing Res Rev*. 2009 Oct;8(4):251-67.
8. Mudera V, Smith AS, Brady M, Lewis MP. The effect of cell density on the maturation and contractile ability of muscle derived cells in a 3D tissue-engineered skeletal muscle model and determination of the cellular and mechanical stimuli required for the synthesis of a postural phenotype. *J Cell Physiol*. 2010 Jun 7;225:646-53.
9. Dennis RG, Kosnik PE, 2nd. Excitability and isometric contractile properties of mammalian skeletal muscle constructs engineered in vitro. *In Vitro Cell Dev Biol Anim*. 2000 May;36(5):327-35.
10. Scime A, Rudnicki MA. Anabolic potential and regulation of the skeletal muscle satellite cell populations. *Curr Opin Clin Nutr Metab Care*. 2006 May;9(3):214-9.
11. Stewart CE, Rotwein P. Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. *Physiol Rev*. 1996 Oct;76(4):1005-26.
12. Jacquemin V, Furling D, Bigot A, Butler-Browne GS, Mouly V. IGF-1 induces human myotube hypertrophy by increasing cell recruitment. *Exp Cell Res*. 2004;299(1):148-58.
13. Quinn LS, Anderson BG, Plymate SR. Muscle-specific overexpression of the type 1 IGF receptor results in myoblast-independent muscle hypertrophy via PI3K, and not calcineurin, signaling. *Am J Physiol Endocrinol Metab*. 2007 Dec;293(6):E1538-51.
14. Matheny RW, Merritt E, Zannikos SV, Farrar RP, Adamo ML. Serum IGF-I deficiency does not prevent compensatory skeletal muscle hypertrophy in resistance exercise. *Exp Biol Med (Maywood)*. 2009 Feb;234(2):164-70.

15. Stewart CE, Pell JM. Point:Counterpoint: IGF is/is not the major physiological regulator of muscle mass. Point: IGF is the major physiological regulator of muscle mass. *Journal of Applied Physiology*. 2010;108(6).
16. Flueck M, Goldspink G. Point:Counterpoint: IGF is/is not the major physiological regulator of muscle mass. Counterpoint: IGF is not the major physiological regulator of muscle mass. *J Appl Physiol*. 2010 Jun;108(6):1821-3; discussion 3-4; author reply 33.
17. Sharples AP, Al-Shanti N, Stewart CE. C2 and C2C12 murine skeletal myoblast models of atrophic and hypertrophic potential: relevance to disease and ageing? *J Cell Physiol*. 2010 Oct;225(1):240-50.
18. Stewart CE, Rotwein P. Insulin-like growth factor-II is an autocrine survival factor for differentiating myoblasts. *J Biol Chem*. 1996 May 10;271(19):11330-8.
19. Foulstone EJ, Huser C, Crown AL, Holly JM, Stewart CE. Differential signalling mechanisms predisposing primary human skeletal muscle cells to altered proliferation and differentiation: roles of IGF-I and TNFalpha. *Exp Cell Res*. 2004 Mar 10;294(1):223-35.
20. Florini JR, Ewton DZ, Coolican SA. Growth hormone and the insulin-like growth factor system in myogenesis. *Endocr Rev*. 1996 Oct;17(5):481-517.
21. Sasai N, Agata N, Inoue-Miyazu M, Kawakami K, Kobayashi K, Sokabe M, et al. Involvement of PI3K/Akt/TOR pathway in stretch-induced hypertrophy of myotubes. *Muscle Nerve*. 2010 Jan;41(1):100-6.
22. Hornberger TA, Chu WK, Mak YW, Hsiung JW, Huang SA, Chien S. The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle. *Proc Natl Acad Sci U S A*. 2006 Mar 21;103(12):4741-6.
23. O'Neil TK, Duffy LR, Frey JW, Hornberger TA. The role of phosphoinositide 3-kinase and phosphatidic acid in the regulation of mammalian target of rapamycin following eccentric contractions. *J Physiol*. 2009 Jul 15;587(Pt 14):3691-701.
24. Sun Y, Bilan PJ, Liu Z, Klip A. Rab8A and Rab13 are activated by insulin and regulate GLUT4 translocation in muscle cells. *Proc Natl Acad Sci U S A*. 2010 Nov 1.
25. Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS, Kimball SR. Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. *J Nutr*. 2000 Oct;130(10):2413-9.
26. Anthony JC, Anthony TG, Kimball SR, Vary TC, Jefferson LS. Orally administered leucine stimulates protein synthesis in skeletal muscle of postabsorptive rats in association with increased eIF4F formation. *J Nutr*. 2000 Feb;130(2):139-45.
27. Findlay GM, Yan L, Procter J, Mieulet V, Lamb RF. A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. *Biochem J*. 2007 Apr 1;403(1):13-20.
28. DeYoung MP, Horak P, Sofer A, Sgroi D, Ellisen LW. Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes Dev*. 2008 Jan 15;22(2):239-51.
29. Drummond MJ, Miyazaki M, Dreyer HC, Pennings B, Dhanani S, Volpi E, et al. Expression of growth-related genes in young and older human skeletal muscle following an acute stimulation of protein synthesis. *J Appl Physiol*. 2009 Apr;106(4):1403-11.
30. Spangenburg EE, Le Roith D, Ward CW, Bodine SC. A functional insulin-like growth factor receptor is not necessary for load-induced skeletal muscle hypertrophy. *J Physiol*. 2008 Jan 1;586(1):283-91.



31. Heron-Milhavet L, Mamaeva D, LeRoith D, Lamb NJ, Fernandez A. Impaired muscle regeneration and myoblast differentiation in mice with a muscle-specific KO of IGF-IR. *J Cell Physiol.* 2010 Oct;225(1):1-6.
32. Tatsumi R. Mechano-biology of skeletal muscle hypertrophy and regeneration: possible mechanism of stretch-induced activation of resident myogenic stem cells. *Anim Sci J.* 2010 Feb;81(1):11-20.
33. Yamada M, Tatsumi R, Yamanouchi K, Hosoyama T, Shiratsuchi S, Sato A, et al. High concentrations of HGF inhibit skeletal muscle satellite cell proliferation in vitro by inducing expression of myostatin: a possible mechanism for reestablishing satellite cell quiescence in vivo. *Am J Physiol Cell Physiol.* 2010 Mar;298(3):C465-76.
34. Morgan J, Rouche A, Bausero P, Houssaini A, Gross J, Fiszman MY, et al. MMP-9 overexpression improves myogenic cell migration and engraftment. *Muscle Nerve.* 2010 Oct;42(4):584-95.
35. Armstrong DD, Esser KA. Wnt/beta-catenin signaling activates growth-control genes during overload-induced skeletal muscle hypertrophy. *Am J Physiol Cell Physiol.* 2005 Oct;289(4):C853-9.
36. Gentile MA, Nantermet PV, Vogel RL, Phillips R, Holder D, Hodor P, et al. Androgen-mediated improvement of body composition and muscle function involves a novel early transcriptional program including IGF1, mechano growth factor, and induction of {beta}-catenin. *J Mol Endocrinol.* 2010 Jan;44(1):55-73.
37. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, et al. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nature genetics.* 2006 Feb;38(2):228-33.
38. McCarthy JJ, Esser KA. Anabolic and catabolic pathways regulating skeletal muscle mass. *Curr Opin Clin Nutr Metab Care.* 2010 May;13(3):230-5.
39. Glass D, Roubenoff R. Recent advances in the biology and therapy of muscle wasting. *Ann N Y Acad Sci.* 2010 Nov;1211:25-36.
40. McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature.* 1997 May 1;387(6628):83-90.
41. McPherron AC, Lee SJ. Double muscling in cattle due to mutations in the myostatin gene. *Proc Natl Acad Sci U S A.* 1997 Nov 11;94(23):12457-61.
42. Lee SJ. Sprinting without myostatin: a genetic determinant of athletic prowess. *Trends Genet.* 2007 Oct;23(10):475-7.
43. Wagner KR, Fleckenstein JL, Amato AA, Barohn RJ, Bushby K, Escolar DM, et al. A phase I/II trial of MYO-029 in adult subjects with muscular dystrophy. *Ann Neurol.* 2008 May;63(5):561-71.
44. Castro-Gago M, Blanco-Barca MO, Eiris-Punal J, Carneiro I, Arce VM, Devesa J. Myostatin expression in muscular dystrophies and mitochondrial encephalomyopathies. *Pediatr Neurol.* 2006 Apr;34(4):281-4.
45. Rios R, Fernandez-Nocelos S, Carneiro I, Arce VM, Devesa J. Differential response to exogenous and endogenous myostatin in myoblasts suggests that myostatin acts as an autocrine factor in vivo. *Endocrinology.* 2004 Jun;145(6):2795-803.
46. Langen RC, Van Der Velden JL, Schols AM, Kelders MC, Wouters EF, Janssen-Heininger YM. Tumor necrosis factor-alpha inhibits myogenic differentiation through MyoD protein destabilization. *Faseb J.* 2004 Feb;18(2):227-37.
47. McFarlane C, Plummer E, Thomas M, Hennebry A, Ashby M, Ling N, et al. Myostatin induces cachexia by activating the ubiquitin proteolytic system through an

- NF-kappaB-independent, FoxO1-dependent mechanism. *J Cell Physiol.* 2006 Nov;209(2):501-14.
48. Trendelenburg AU, Meyer A, Rohner D, Boyle J, Hatakeyama S, Glass DJ. Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size. *Am J Physiol Cell Physiol.* 2009 Jun;296(6):C1258-70.
  49. Tsuchida K, Nakatani M, Uezumi A, Murakami T, Cui X. Signal transduction pathway through activin receptors as a therapeutic target of musculoskeletal diseases and cancer. *Endocr J.* 2008 Mar;55(1):11-21.
  50. Rebbapragada A, Benchabane H, Wrana JL, Celeste AJ, Attisano L. Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. *Mol Cell Biol.* 2003 Oct;23(20):7230-42.
  51. Sartori R, Milan G, Patron M, Mammucari C, Blaauw B, Abraham R, et al. Smad2 and 3 transcription factors control muscle mass in adulthood. *Am J Physiol Cell Physiol.* 2009 Jun;296(6):C1248-57.
  52. Welle SL. Myostatin and muscle fiber size. Focus on "Smad2 and 3 transcription factors control muscle mass in adulthood" and "Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size". *Am J Physiol Cell Physiol.* 2009 Jun;296(6):C1245-7.
  53. Cong H, Sun LQ, Liu C, Tien P. Inhibition of atrogen-1/MAFbx expression by adenovirus- delivered shRNAs attenuates muscle atrophy in fasting mice. *Hum Gene Ther.* 2010 Dec 2.
  54. Whittemore LA, Song K, Li X, Aghajanian J, Davies M, Girgenrath S, et al. Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. *Biochem Biophys Res Commun.* 2003 Jan 24;300(4):965-71.
  55. Lee SJ, Reed LA, Davies MV, Girgenrath S, Goad ME, Tomkinson KN, et al. Regulation of muscle growth by multiple ligands signaling through activin type II receptors. *Proc Natl Acad Sci U S A.* 2005 Dec 13;102(50):18117-22.
  56. McFarlane C, Hennebry A, Thomas M, Plummer E, Ling N, Sharma M, et al. Myostatin signals through Pax7 to regulate satellite cell self-renewal. *Exp Cell Res.* 2008 Jan 15;314(2):317-29.
  57. Leger B, Derave W, De Bock K, Hespel P, Russell AP. Human sarcopenia reveals an increase in SOCS-3 and myostatin and a reduced efficiency of Akt phosphorylation. *Rejuvenation research.* 2008 Feb;11(1):163-75B.
  58. Amthor H, Macharia R, Navarrete R, Schuelke M, Brown SC, Otto A, et al. Lack of myostatin results in excessive muscle growth but impaired force generation. *Proc Natl Acad Sci U S A.* 2007 Feb 6;104(6):1835-40.



## Figure Legend

Figure 1. The regulation of protein synthesis and muscle hypertrophy vs. protein degradation and muscle atrophy.

IGF-I; Insulin-like growth factor-I, IRS-1; Insulin Receptor Substrate-1, TNF- $\alpha$ ; Tumour Necrosis Factor-Alpha, ALK 4, 5; Activin Receptor-Like-Kinase 4 and Activin Receptor-Like-Kinase 5, ECM; Extracellular Matrix, HGF; Hepatocyte Growth Factor, TRADD; TNF Receptor 1 Associated Death Domain, TRAFF; TNF receptor Associated Factor, FAK; Focal Adhesion Kinase, FADD; Fas Associated Death Domain, FOXO; Forkhead Homeobox Type O, PDK-1; Phosphoinositide-Dependent Kinase-1, Akt; Protein Kinase B, MAPK; Mitogen Activated Protein Kinase, mTOR; Mammalian Target of Rapamycin, PI3K; Phosphatidylinositol 3-kinase, ERK; Extra Cellular Signal Regulated Kinase, Ras; Ras Protein, Raf; MAP Kinase Kinase Kinase (MAP3K), p70S6K; P70S6 Kinase- a serine/threonine kinase- Phosphorylation of S6 induces protein synthesis at the ribosome, TSC1, TSC2; Tuberous Sclerosis Protein 1, Tuberous Sclerosis Protein 2, Redd 2; Regulated in Development and DNA Damage Response 2, Rheb; Ras Homolog Enriched in Brain, PLD; Phospholipase D, PA; Phosphatidic acid PA, I- $\kappa$ B $\alpha$ ; Inhibitor-Kappa B Alpha, NF- $\kappa$ B; Nuclear Factor-Kappa B,  $\beta$ -Catenin; Beta-Catenin.